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Multimetal Resistance Potential of Indigenous Bacterial Genera of Sea Shore Soils of Andaman Islands of India

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Authors' contributions

This work was carried out in collaboration between all authors. Author PD designed the study with suggestions from author MB, wrote the protocol and wrote the first draft of the manuscript. The AAS study for soil samples done by author DH. Both authors PD and DH managed the literature searches. Author MB edited the whole manuscript to attain its final version. All authors read and approved the final version of the manuscripts.

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ABSTRACT

Aims: Isolation and characterization of heavy metal tolerant microorganism from sea shore soil of Andaman Islands (India).
Study Design:
(a) Sample collection and isolation of multimetal resistant bacteria.
(b) Relative growth of bacterial isolates in presence of heavy metal.
(c) Characterization of bacterial isolates and antibiotic sensitivity profile.
(d) Capability of the production of extracellular enzyme(s).
Place and Duration of Study: Sea shore soils were collected from Ross I, Ross II, Port Blair and Havelock of Andaman Islands (India) with seasonal variation.
Methodology: Soil samples were analyzed for physico-chemical and microbiological characteristics. Bacteria isolated from sea shore soil were tested for their ability to tolerate cadmium, chromium, zinc, nickel, copper, cobalt, manganese, mercury, lead and arsenic in their

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growth medium and their relative growth in the presence of heavy metals determined. Antibiotic susceptibility test was done for different antibiotics with different concentrations. Biochemical tests were done to observe the diversity of the isolates. Degree of NaCl tolerance and extracellular enzyme production of selected isolates were done.

Results: The abundance of heavy metals in the sea shore soil are Pb>Zn>Cd>Cu>Mn>Ni=Co>Cr. The relative growth of the bacterial isolates were different for each strain, but the general order of resistance to the metals supplemented media was found to be as Pb> As> Mn> Zn > Cu > Cd> Ni and toxic effects of these metals increased with increasing concentration; however most of the isolates were sensitive to Hg, Cr and Co. Antibiotic susceptibility test showed varying results. Additionally, it was found that the strains were sensitive to four of the antibiotics tested. Biochemical characterization was indicative of the diverse microbial flora having multimetal resistance on one hand; on the other hand, they are potent producers of many useful enzymes like amylase, protease, lipase, catalase, urease, phosphatase etc., and are moderately halophilic.

Conclusion: The bacterial isolates from saline soil are of interest as they exhibit profound heavy metal tolerance and hence may be promising for bioremediation purpose and their molecular mechanisms for resistance to multiple metals needs further speculation.

Keywords: Andaman; antibiotic; bioremediation; multimetal resistant.

1. INTRODUCTION

Heavy metals are natural chemical elements and components of the earth's crust that are characterized by relatively high density and high relative atomic weight. Usually their atomic number is greater than 20 [1,2]. Although heavy metals are naturally present in the environment, their presence as a contaminant in ecosystems results mainly from both natural and anthropogenic activities [2]. Heavy metals are very toxic to the human body because they interfere with the normal biochemical reactions of the body. Some heavy metals such as Co, Cu, Fe, Mn, Mo, Ni, V and Zn are required in minute quantities by organisms. However, higher concentrations of these metals often are cytotoxic. Other heavy metals such as Pb, Cd, Hg, Cr and As have been known to be extremely toxic at lower concentrations [3], although, they have no significant biological function so far reported and are thus regarded as the "main threats" since they are very harmful to both plants and animals [2].

The release of heavy metals into the environment causes an environmental pollution problem because they are non-degradable normally and accumulate in living organisms. Soil and water are usually considered as the ultimate fate for heavy metals, however, until recently, relatively little has been known regarding the way heavy metals are bound to soils or precipitate in water and the ease with which they may be released [4]. Since they are non-degradable in the environment they are constantly recycled under the influence of natural processes such as weathering, erosion or biological activity. Various strategies have been developed to solve the problem; among them chemical precipitation or solvent extraction has been employed to precipitate the metal [5] but it is difficult to apply on large surface [6], is very expensive and generates secondary products.

Although high concentrations of heavy metals have a negative impact on microbial communities in the metal polluted environment, but some bacteria can tolerate or even proliferate in the presence of specific metals [4,5,6,7,8]. Bacterial tolerance relative to heavy metals may be defined as the ability of the bacteria to cope with metal toxicity by detoxifying mechanisms which are activated as a result of the presence of the specific metals [9,10]. But at very high concentration heavy metals have been reported to inhibit bacterial growth [11]. Metal uptake by microorganisms depends on the characteristics of metal ions, surface features of the microorganisms, cell physiology and physicochemical influences from the environment, e.g. pH, temperature and metal concentration of environment [12]. Microorganisms which have the ability to survive in highly concentrated heavy metals can be used as an agent of bioremediation. The process of bioaccumulation is easily performed which is based on the incorporation of metals inside the biomass that absorbs the metal ions at the cellular surface through various mechanisms. Microorganisms resistant to both metals and antibiotics, isolated frequently from different environments [13,14] are caused by selection resulting from metals present in the particular

environment [15,16] and can pose a public health risk [17].

Metal bioaccumulation by marine organisms has been the subject of considerable interest in recent years because of serious concern that high levels of metals may have detrimental effects on the marine organisms and may create problems in relation to their suitability as food for humans. Compared to sediments, marine organisms exhibit greater spatial sensitivity and therefore, are the most reliable tool for identifying sources of biologically available heavy metal contamination [18].

The objective of this study was to isolate and identify multi-metal tolerant bacteria from sea shore soil to evaluate their ability to tolerate different concentrations of mercury, cadmium, zinc, nickel, cobalt, copper, arsenic, lead, cadmium and chromium so that they can be efficiently used as bioremedial tools.

2. MATERIALS AND METHODS

2.1 Sample Collection and Bacterial Culture Isolation

Soil samples were collected in sterile falcon tubes from three sites of Andaman Island, India. After collection, soil samples were stored at 4° C until further characterization. 1 gm of soil sample were serially diluted and 0.1 ml of diluted sample was spread over the LA plate and incubated at 37℃ for 24 - 48 hr. Bacterial colonies were picked out and purified and maintained in LA slant.

2.2 Physico-chemical Analysis of Soil Samples

The soil samples were dissolved in 1(N) KCl in the ratio of 1:2.5, and the mixture was allowed to shake for 1 hr; the pH was estimated using digital pH meter. Soil EC, salinity, TDS were determined by suspending the air dried sample in the ratio of 1:2 after shaking the mixture overnight, it was filtered and the filtrate was analyzed using digital conductivity meter (Wenser, Model: LMMP-30). The total concentration of heavy metals was estimated by digestion of 1 gm air dried soil in 10 mL of HNO_3: HCl (1:3) and digested samples were transferred into 50 ml micro Kjeldahl flask. Soil was subjected to acid digestion using standard method [19] and the concentrations of Co, Cr, Cu, Cd, Mn, Ni, Pb and Zn were determined by atomic absorption spectroscopy (Agilent spectra).

2.3 Primary Screening

Qualitative assessment of the metal resistance of the isolates was made following the method of Abbas and Edward [20]. The isolates were grown on Luria agar medium which was supplemented with 100 to 400 µg/ml of Zn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, 25 to 200 µg/ml of Cr^{2+}, Cu^{2+}, Mn^{2+} and 10 to 40 µg/ml of Hg^{2+}, As^{3+}, Pb^{2+}. Overnight culture was streaked in the form of a narrow line on metal incorporated plates and incubated at 37^0C for 48 hours for visible growth.

2.4 Secondary Screening

The degree of resistance of the selected isolates was also evaluated in the LB medium. Sterilized metal solutions were added to the sterilized LB medium to attain metal concentrations ranging from 25 to 75 µg/ml. Growth of the isolates was determined as optical density at 540 nm and the relative growth was expressed as a percentage of those obtained in untreated control cultures at the same time which was taken as 100%.

2.5 Isolation of Plasmid DNA

Isolates were screened for the presence of plasmids by alkaline lysis method [21].

2.6 Biochemical Characterization of Isolates

The bacterial isolates were characterized biochemically by Gram nature, carbohydrate utilization, denitrification test, arginine dehydrolase test, IMViC test, gelatin, oxidase, urease, catalase test and H_2S production. Hi Assorted Biochemical test kit (Himedia) was used for Gram -ve isolates. Standard biochemical procedure [22] and HiDtect Carbohydrate Fermentation Disc (Himedia) was used for Gram + ve isolates.

2.7 Resistance to Antibiotics

To determine the antibiotic sensitivity of the metal resistant isolates , antibiotic impregnated discs (G-I-Minus and G-VIII-Plus discs; Himedia) [Ampicillin (10 mcg), Tetracycline (5mcg), Penicillin-G (10 mcg), Streptomycin (10 mcg), Gentamycin (10 mcg), Polymixin B (300 mcg),

Chloramphenicol (30 mcg), Co-Trimoxazole (25 mcg), Nitrofurantoin (300 mcg)] were placed on freshly prepared lawns of each isolates on LA plates. Plates were incubated at 37℃ for 24 hr. Depending on inhibition zones, the isolates were categorized as sensitive, intermediate and resistant as per manufacturer manual.

2.8 NaCl Tolerance

Degrees of NaCl tolerance of selected isolates were evaluated in Luria broth containing 5%, 10% and 20% NaCl. Growth of the isolates in LB was determined by measuring the optical density at 540 nm using the uninoculated broth as blank. The relative growth of the isolates was expressed as the percentage of those obtained in untreated control which was taken as 100%.

2.9 Extracellular Enzymatic Activity

Extracellular enzyme production was determined by standard method [22]. Isolates streaked on test agar medium with respective substrates such as starch, calcium phosphate, tributyrin, casein agar plates separately and incubated at 37℃ for 24-48 h. After incubation, plates were flooded with respective indicator solution and the development of clear zone around the growth of the organism was documented as positive results for enzyme activity.

3. RESULTS AND DISCUSSION

Presence of metal tolerant bacterium in a given environment may have resulted from increasing environmental pollution and may be an indication that such area is affected by heavy metals.

Physico-chemical parameters such as pH, temperature, electrical conductivity, salinity and TDS of soil samples were measured and are listed in Table 1. Maximum EC (5.89 µS/cm) and salinity (3.22 psu) was found in Hv (Table 1). It was observed that Cu was the most abundant metal in the soils, while Cr and Cd showed lower levels (Table 2). According to heavy metal profile RsII and Hv are more toxic samples.

Heavy metal resistant bacteria were isolated from samples collected from sea shore environment of four different regions of Andaman Islands. Total bacterial count were found as $2X10^3$, $0.4x10^2$, $1.8x10^2$, $0.6x10^2$ CFU/gm of soil from RsI, RsII, Hv and Po sites respectively (Table 3).

44 different bacterial colonies were isolated from the samples collected from four different sites. Among them, 23 isolates showed growth almost equivalent to control in different heavy metal incorporated plates (100 µg/ml of Zn^{2+},Ni^{2+}, Co^{2+}, Cd^{2+}; 25 µg/ml of Cr^{2+},Cu^{2+}, Mn^{2+}; 10 µg/ml Hg^{2+} and 40 µg/ml of As and Pb). Higher concentrations of metals, however inhibited the growth of all the isolates (Fig. 1).

The selected 23 isolates showed fairly high tolerance to 10 different metals were subjected to liquid screening in metal incorporated LB media. Optical density at 540nm was measured. Relative growth (%) was calculated with respect to control (Table 4).

Table 1. Physico-chemical analysis of soil sample

Sampling site	Sample name	pH	Temp*	Salinity*	TDS*	EC*	Av N	Av P	Av K
Ross Island I	RsI	8.2	22	0.60	598	0.18	78.4	2.688	84.56
Ross Island II	RsII	7.93	20	2.17	2.07	4.13	47.04	3.136	195.104
Havelock Island	Hv	8.0	21	3.22	2.07	5.89	282.2	2.912	242.816
Portblair	Po	7.96	22	2.80	2.60	5.21	15.68	2.912	288.4

**Temperature- ℃, EC-Electrical conductance (mS/cm), Salinity-Practical salinity units (psu), TDS- Total dissolved solids (ppm), Av N —Available nitrogen (Kg/ha), Av P — Available phosphorus (Kg/ha), Av K— Available potassium (Kg/ha)*

Table 2. Heavy metal analysis of the soil samples

Sample	Metals (µg/mg of soil)							
	Cd	Pb	Mn	Ni	Zn	Cu	Cr	Co
RsI	0.022	0.072	0.08	0.02	0.04	0.12	0	0.03
RsII	0.009	0.079	0.08	0.03	0.03	0.12	0.008	0.03
Hv	0.008	0.078	0.02	0.03	0.02	0.11	0.008	0.02
Po	0.008	0.084	0.03	0.03	0.03	0.12	0	0.03

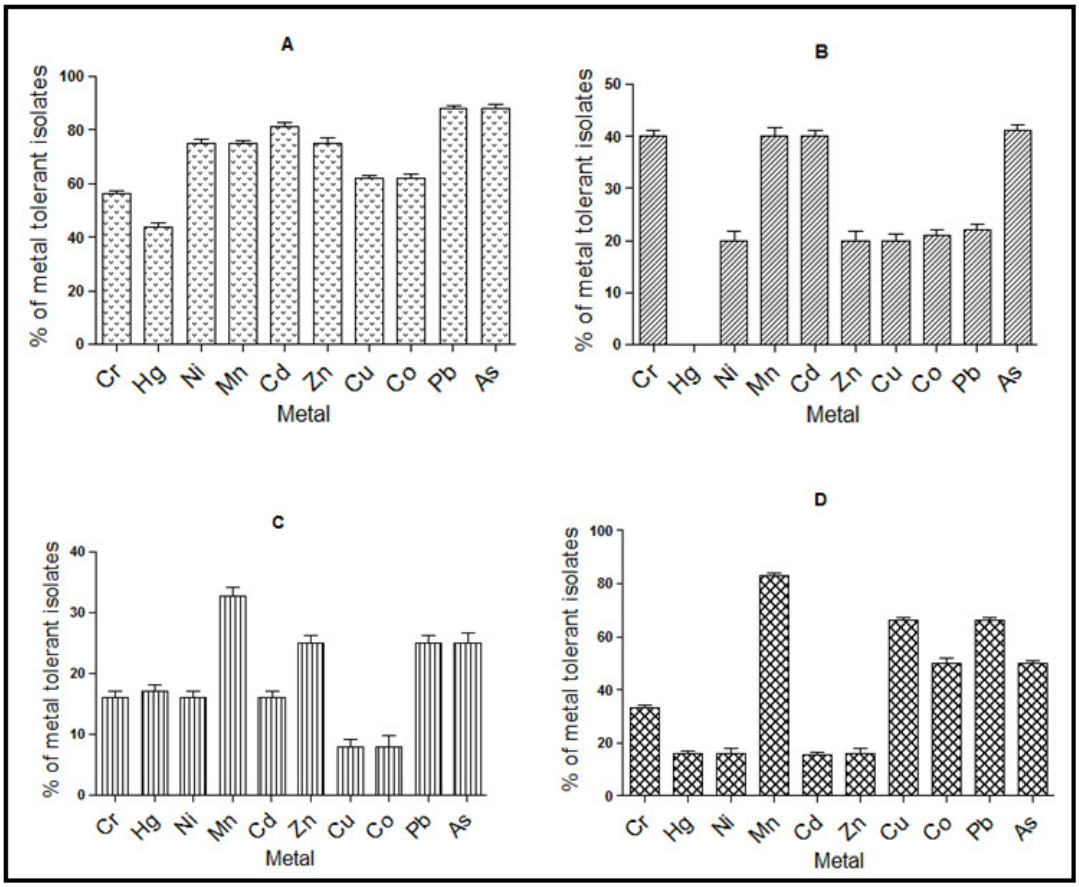

Fig. 1. Metal tolerance of soil isolates in solid medium (A-RsI, B-RsII, C- Hv and D- Po)

Table 3. Isolation of bacteria from various location of Andaman

Sample name	Number of normal flora (CFU/gm)
RsI	$2x10^3$
RsII	$0.4x10^2$
Hv	$1.8x10^2$
Po	$0.6x10^2$

Nine isolates showed ≥ 50% relative growth in LB at 50 µg/ml of most of the tested metals except Hg, Cr and Co. The performance of the isolates in liquid salts medium with 75 µg/ml of metal was very poor. None of the isolates examined showed detectable growth at 100 µg/ml. Mercury was by far more toxic. The order of the toxicity of the metals tested were Hg^{2+}>Cr^{6-} >Co^{2+}> Ni^{2+}>Cd^{2+}>Cu^{2+}>Zn^{2+}>Mn^{2+}>As^{3+}>Pb^{2+}.

From the present study it has been found that RsIL1, RsIL8, RsIL10, RsIL11, RsIL12, RsIL13, RsIIL6, HvL0 and PoL10 isolates were resistant to lead and RsIL1, RsIL10, RsIL11, RsIL12, RsIL13 and RsIIL6 were resistant to arsenic**.** The resistance to lead and arsenic could be attributed to the high lead and arsenic content of the soil [23]. Increased industrialization has resulted in environmental contamination by nickel in many aquatic systems. RsIIL6 and HvL0 demonstrated resistance to nickel. Therefore, the nickel-resistant isolates could be an interesting tool as an environmental marker [24]. The greater affinity of NaCl for Zn has been reported due to the formation of a soluble zinc-chloro complex. Hence the greater resistance of the isolates for Zn could be attributed to the fact that such strains being of marine environment possess higher tolerance level. However, in this study resistance to lower concentration of Cr and Co was observed and isolates were highly susceptible to Hg, suggesting that the contamination of these metals in these soils could be low [25].

Table 4. Metal tolerance of some selected bacterial isolates

Isolates	Relative growth (%)						
	Metal in medium (50 µg/ml)						
	Pb	As	Mn	Zn	Cu	Ni	Cd
RsIL1	83	93	90	86	22	16	40
RsIL8	96	0	82	56	0	18	48
RsIL10	78	94	83	46	76	1	56
RsIL11	76	89	81	59	1	28	44
RsIL12	83	79	81	75	88	18	57
RsIL13	63	90	92	45	58	2	57
RsIIL6	51	84	64	64	66	76	17
HvL10	70	12	58	56	62	73	31
PoL10	64	5	90	47	23	16	40

Plasmid profile of isolates exhibited a single band with the size of around 15 kb in 6 isolates (RsIL1, RsIL8, RsIL10, RsIL11, RsIL12 and RsIL13) which indicates the presence of mega plasmid (Fig. 2).

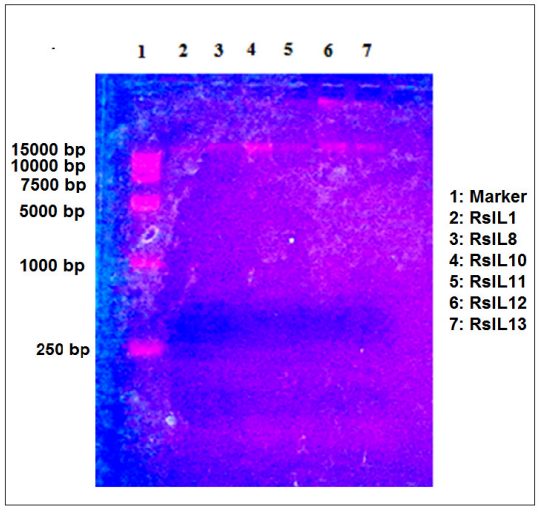

Fig. 2. Plasmid profile of isolates

The resistance to a particular heavy metal has been correlated to antibiotics and other heavy metal resistance in a variety of organisms [26,27,28] and the role of plasmids in conferring resistance to both antibiotics and metals has been previously demonstrated [29]. Bacteria exposed to high levels of heavy metal in their environment have adapted to this stress by developing various resistance mechanisms. Heavy metals resistant traits are often carried by plasmids [28,30,31]. Such bacteria could be utilized for detoxification and removal of heavy metals from contaminated environment.

Among nine isolates, four were gram positive (RsIL1, RsIL11, RsIL13, RsIIL6) and five were gram negative (RsIL8, RsIL10, RsIL12, HvL10 and PoL10). All isolates showed good growth in the presence of glucose and xylose. Maximum isolates showed positive results for urease, catalase, and good growth in presence of sorbitol. All isolates were negative in case of H_2S production, gelatin hydrolysis, indole and methyl red production and Voges-Proskauer test. Only gram negative isolates were characterized biochemically by lysine utilization, ornithine utilization, and phenylalanine deaminase. All gram negative isolates showed negative responses to lysine utilization, ornithine utilization and phenylalanine deamination (Table 5).

Antibiotic sensitivity profiles of the isolates have indicated that, maximum isolates were resistant to co-trimoxazole and nitrofurantoin (Table 6) whereas all isolates were highly sensitive to streptomycin, ciprofloxacin, gentamycin and tetracycline. Ampicillin resistant isolates were RsIL1, RsIL12 and HvL10. Only RsIL13 showed intermediate response in case of penicillin. Resistance to heavy metal is often associated with plasmids, which also encodes resistance to antibiotics [26,27]. Here isolates from Andaman sea shore exhibited maximum resistance to ampicillin, co-trimoxazole and nitrofurantoin unlike as shown by a different group dealing with sea shore microbial isolates which exhibited resistance to ampicillin, tetracycline, streptomycin and gentamycin [28]. Although in this study correlation between metal and antibiotic resistance has not been studied yet it is already reported that heavy metal induced antibiotic resistance might be ubiquitous among various microbial species. This might play a role in the emergence and spread of antibiotic resistance in metal and antibiotic co-contaminated environments [32].

Isolates have been categorized as slightly, moderately and extremely halophilic on the basis of tolerance to different concentration of NaCl. From the relative growth in LB incorporated with sodium chloride it is evident (Fig. 3) that the growth of the isolates decreased gradually with the increase in concentration of NaCl. 50-60% relative growth was observed in medium supplemented with 5% NaCl; growth was further reduced to around 5-10% with 10% NaCl; However RsIL10 and RsIL12 significantly showed comparable growth both in 10% and 20% NaCl unlike other isolates whose growth was drastically reduced with increase in sodium chloride concentration. On the basis of relative

growth in presence of NaCl, all isolates have been categorized as moderately halophilic.

Since marine isolates have been known to produce extracellular enzymes preliminary screening was done to determine the potential of the selected strains for enzyme production like amylase, phosphatase, lipase and protease (Fig. 4). It was found that 89% bacterial isolates produce phosphatase, 55% produce amylase, 22% produce lipase and 11% produce protease.

It has been studied that halophilic bacteria are often potent producers of many useful enzymes like amylase, protease, lipase, catalase, urease, phosphatase etc [33]. These enzymes are attractive for industrial uses. It is important that enzyme properties may be improved by the use of protein engineering technique. The possibility to have a wide variety of moderate halophiles producing extracellular enzymes will be of invaluable tool for industrial and biotechnological applications.

Table 5. Morphological and biochemical characterization of bacterial isolates

Tests	Isolates								
	RsIL1	**RsIL11**	**RsIL13**	**RsIIL6**	**RsIL8**	**RsIL10**	**RsIL12**	**HvL10**	**PoL10**
Gram character	(+) ve	(+) ve	(+) ve	(+) ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
Morphology	Rod	Rod	Cocci	Cocci	Cocci	Cocci	Cocci	Rod	Rod
CI	(-)ve	(+)ve	(+)ve	(-)ve	(-)ve	(+)ve	(+)ve	(-)ve	(-)ve
UR	(+)ve	(+)ve	(+)ve	(-)ve	(+)ve	(+)ve	(+)ve	(+)ve	(-)ve
DN	(+)ve	(+)ve	(-)ve	(-)ve	(-)ve	(+)ve	(+)ve	(+)ve	(-)ve
HP	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
GL	(-)ve	(-)ve	(-)ve	(-)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve
GG	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve
GS	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(-)ve	(+)ve	(-)ve	(-)ve
GX	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve
GM	(-)ve	(-)ve	(-)ve	(+)ve	(+)ve	(-)ve	(+)ve	(-)ve	(+)ve
GH	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
CA	(+)ve	(+)ve	(+)ve	(-)ve	(+)ve	(+)ve	(+)ve	(-)ve	(+)ve
IN	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
MR	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
VP	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
LU	ND	ND	ND	ND	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
OU	ND	ND	ND	ND	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
PD	ND	ND	ND	ND	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve

** GG- Growth in glucose, GL- Growth in Lactose, GS-Growth in Sucrose, DN- Denitrification, AD- Arginine dehydrolase, IN- Indole, MR- Methyl red, VP- Voges-Proskauer, CI- Citrate, GH- Gelatin hydrolysis, SH- Starch hydrolysis, LH- Lipid hydrolysis, CH- Casein hydrolysis, OX- Oxydase, UR- Urease, CA- Catalase, HP-H_2S production, PD-Phenylalanine Deaminase, OU- Ornithine utilization, LU- Lysine utilization, GX-Growth in Xylose, GM-Growth in Mannitol, GAD-Growth in Adonitol, GA-Growth in Arabinose, GS-Growth in Sorbitol.*

Table 6. Antibiotic sensitivity profile of metal resistant isolates

Isolates	Resistant	Intermediate	Sensitive
RsIL1	C ,Cot, Amp, Nit, P		CIP, Te, S, Gen
RsIL8	Cot, Nit, P		CIP, Tet, S, C, Amp, Gen
RsIL10	C, Cot, Nit, P		CIP, Tet, S, Amp, Gen
RsIL11	Cot, Nit		CIP, Tet, S, C, Amp, Gen
RsIL12	Cot, Amp, Nit, P		CIP, Tet, S, C, Gen
RsIL13	Cot, Nit	P	CIP, Tet, S, C, Amp, Gen
RsIIL6			CIP, Tet, S, C, Amp, Gen, Cot, Nit, P
HvL10	Amp, Nit, P		CIP, Tet, S, C, Gen, Cot
PoL10	Amp		CIP, Tet, S, C, Gen, Cot, Nit, P

**Amp-Ampicillin (10 mcg), Te-Tetracycline (5 mcg), P-Penicillin-G (10 mcg), S-Streptomycin (10 mcg), Gen-Gentamycin (10 mcg), PB-Polymixin B (300 mcg), C-Chloramphenicol (30 mcg), Cot-Co-Trimoxazole (25 mcg), Nit-Nitrofurantoin (300 mcg)*

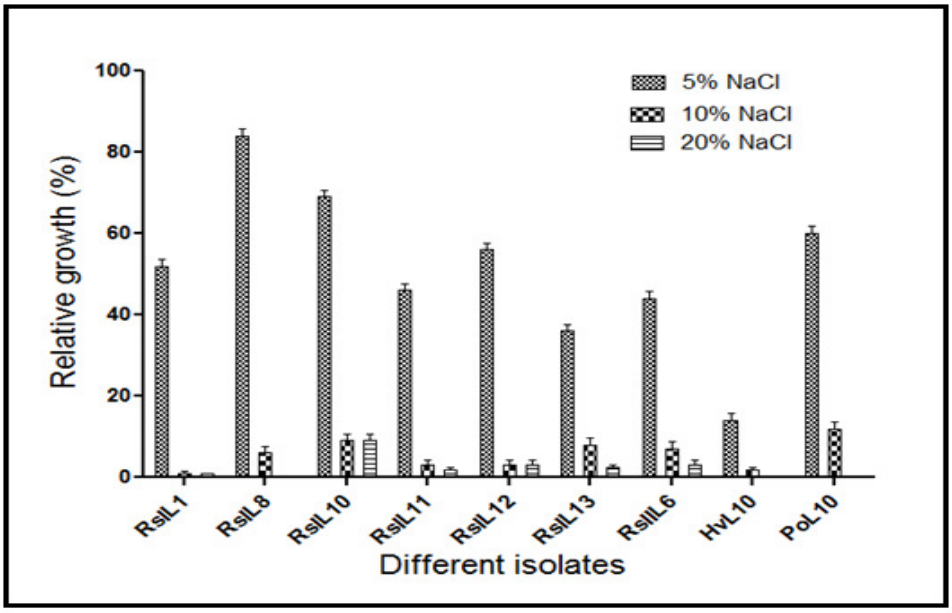

Fig. 3. NaCl tolerance of bacterial isolates

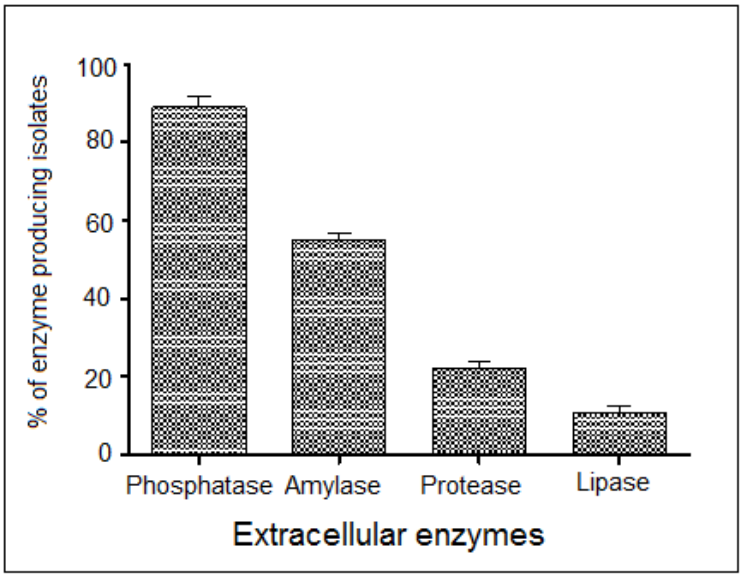

Fig. 4. Extracellular enzyme production by selected isolates

4. CONCLUSION

From the above study it can be concluded that the relative growth of isolates in presence of heavy metals were different for each strain but the general order of resistance to the metals was found to be as Pb> As> Mn> Zn > Cu > Cd> Ni. The toxic effects of the metals increased with increasing concentrations and the strains were resistant to at least one antibiotic except RsIIL6. Isolates were moderately halophilic and produced extracellular enzymes. All these results suggest that the isolates could survive in heavy metal contaminated sediments. Therefore, the isolates may be useful as indicators of potential toxicity of heavy metals in coastal area and they could be designed as bioremediation tools [34,35,36].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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